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14. ABSTRACT Real-time monitoring of human performance biomarkers for fatigue, vigilance, and stress among DoD personnel during key missions requires devices that enable facile detection of multiple targets with minimal user intervention. Since the biomarkers are present at dilute levels, pre-concentration under electrical and magnetic fields within microfluidic devices is needed to locally enrich the concentration of each relevant target, so that they may be detected within biofluids that contain high levels of interfering molecules. Optimized conditions of these force fields can then be routinely applied within field settings for facile electrical and optical detection, with minimal user intervention. In this project, researchers developed nano-slit devices and optimized electrokinetic pre-concentration conditions for key neurological biomarkers of interest, by using nanoparticles and aptamers to enhance specificity. Additionally, pre-concentration was coupled to various detection paradigms to achieve high-sensitivity biomarker profiles for future application towards unraveling the signaling pathways for assessing and mitigating stress.					
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Final Report for AOARD Grant #114083 “Nanofluidic devices for enhancing detection sensitivity and selectivity of biomarkers for human performance monitoring”

November 24, 2014

PI and Co-PI information: Nathan S. Swami; nswami@virginia.edu; University of Virginia; Department of Electrical & Computer Engineering; Charlottesville, VA 22904; Phone: (434) 924 1390; Fax: (434) 924 8818.

Co-PI: Chia-Fu Chou; cfchou@phys.sinica.edu.tw; Academia Sinica, Taiwan

Period of Performance: 08/09/2011 – 08/08/2014

Abstract: The real-time monitoring of human performance biomarkers for fatigue, vigilance, and stress among DoD personnel during key missions requires devices that enable facile detection of multiple targets with minimal user intervention. Since the biomarkers are present at dilute levels, preconcentration of biomarkers under electrical and magnetic fields within microfluidic devices is needed to locally enrich the concentration of each relevant target, so that they may be detected within biofluids that contain high levels of interfering molecules. The optimized conditions of these force fields can then be routinely applied within field settings for facile electrical and optical detection, with minimal user intervention. In this project, we developed nano-slit devices and optimized the electrokinetic preconcentration conditions for key neurological biomarkers of interest, by using nanoparticles and aptamers to enhance specificity. Additionally, biomarker preconcentration was coupled to various detection paradigms to achieve high-sensitivity biomarker profiles for future application towards unraveling the signaling pathways for assessing and mitigating stress.

Introduction: Assessment and enhancement of the capabilities and alertness of the largest asset of the Air Force, namely their field personnel, is a key vision within the AFOSR and the Human Performance Wing of AFRL¹. Implementation of this vision requires the development of technologies for real-time monitoring of human performance biomarkers for fatigue, vigilance, and stress through micro-sampling from biofluids such as saliva, sweat, blood, and urine. However, characterizing and regulating stress conditions through biomarker expression analysis is a particularly challenging task due to the involvement of multiple inter-related targets with complex inter-regulating circuitry. Additionally, the biomarkers are typically present over a wide concentration range (mg/mL – pg/mL) within biofluids², thereby requiring the application of selective pre-concentration approaches for analyte enrichment over interfering proteins and small molecules. Herein, we seek to develop biomarker preconcentration methodologies based on electrical or magnetic force fields within micro/nanofluidic devices^{3,4} to achieve rapid localized biomarker enrichment due to the ensuing volume reduction (**Fig. 1**). As part of this initiative, this particular collaboration between Nathan S. Swami (Virginia) and Taiwan group led by Chia-Fu Chou (Academia Sinica) seeks to develop preconcentration and detection methodologies based on biomarkers from AFRL’s 711th Human Performance Wing (Nancy Kelley-Loughnane). Specific aims include:

Aim 1: Develop nano-slit device platform for applying AC electrokinetic modalities for frequency-selective biomarker preconcentration

Aim 2: Develop nanoparticle and aptamer-based approaches for enhancing specificity of biomarker preconcentration

Aim 3: Develop approaches for coupling biomarker preconcentration with detection

In this manner, through analyzing the time evolution profiles of nueropeptide biomarkers on this device platform, it is envisioned that the signaling pathways for assessing and mitigating stress can be unraveled for future application towards characterizing vigilance, Traumatic Brain Injury and Post –Traumatic Stress Disorders (PTSD).

Experiment: Towards addressing AFRL’s grand challenge of enabling Human Performance Monitoring, the experimental program was based on biomarkers, nanoparticles and assay chemistries from AFRL’s 711th Human Performance Wing (Jorge Chavez & Nancy Kelley-Loughnane), detection systems from N. Swami’s group (Virginia) and device technologies enabled by the group of C-F. Chou (Academia Sinica). Table 1 describes the experimental organization and supported researchers on this collaborative work.

Project	Supported Personnel	(Location) Outcome ^{citation}
Device for AC electrokinetic preconcentration of biomarkers and nanoparticles	Student: W. Varhue (50%); Postdoc: K.T. Liao (50%) (Year 1)	(Virginia & Taiwan) Nano-slit device with constrictions to frequency-selective enrichment ^{5, 6}
Modifying device fabrication for coupling electrochemical detection to preconcentration	Walter Varhue (50%) (Year 2 & 3)	(Virginia) Microfabricated cover-slip with nano-device for electrochemistry ^{7, 8}
Applying prior devices for preconcentration and detection of neuropeptides	Bankim Sanghavi (30%); AFRL collaborator: Jorge B. Chavez (Year 3)	(Virginia & Taiwan) Detection of NPY and Orexin A in various biological matrices ⁹
Electrochemical (EC) assay for aptamer-based detection of cortisol and NPY	Bankim Sanghavi (50%); AFRL collaborator: Jorge B. Chavez (Year 3)	(Virginia) Aptamer-based EC assay for cortisol and NPY detection (in progress)
Functionalization & mapping transcription factor sites	Yih-Li, Lin (50%) – Year 1 & 2	(Taiwan) Highly parallel analysis for resolving protein-binding locations on DNA probes ^{10, 11, 12}
Dielectrophoretic enrichment coupled to Raman spectroscopy	L. Lesser-Rojas (50%) – Year 3	(Taiwan) High sensitivity biomarker detection ¹³

Results and Discussion: The outcomes of the collaborative project are briefly described below:
1. “Nano-constriction device for rapid protein pre-concentration in physiological media by electrokinetic force balance”, K.T. Liao, M. Tsegaye, V. Chaurey, C.F. Chou, N. Swami*. *Electrophoresis* (2012), 33, 1958-1966. Impact Factor =3.3; DOI: 10.1002/elps.201100707

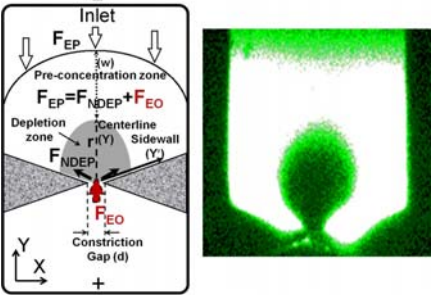


Fig. 1: Biomarker enrichment in physiological media by electrokinetic force fields at nano-constrictions.

2. “Scaling down constriction-based dielectrophoresis for trapping nanoscale biomolecules

Herein, we developed a methodology to steeply enhance biomarker pre-concentration within physiological media over that achieved through negative dielectrophoresis at nanoscale constriction gap devices, by utilizing an additional DC field offset to exponentially enhance the extent of protein depletion across the device. These protein pre-concentration methodologies may be applied towards biomarker discovery, protein crystallization, and rare target sensing for early disease diagnostics.

in high conductivity media”, V. Chaurey, A. Rohani, Y.-H. Su, W. Varhue, K.T. Liao, C. F. Chou, N. S. Swami*, *Electrophoresis* (2013), 34, 1097-1104. Journal Impact Factor =3.3

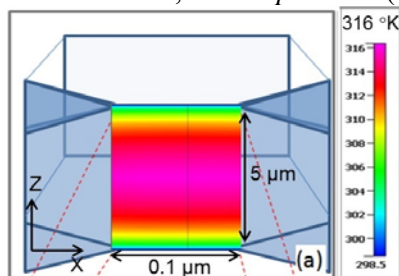


Fig. 2: Temperature rise at nano-Constrictions limits DEP trapping

dielectrophoresis trapping force versus electrothermal drag force on bio-particles.

Selective trapping of nanoscale biomarkers is significant for the separation and high-sensitivity detection of biomarkers. Dielectrophoresis is capable of highly selective trapping of bio-particles based on their characteristic frequency response. However, the trapping forces fall steeply with particle size, especially within physiological media of high-conductivity where the trapping can be dissipated by electrothermal flow due to localized Joule heating. Herein, we investigate the influence of device scaling within the electrodeless insulator dielectrophoresis geometry through the application of highly constricted channels of successively smaller channel depth, on the net balance of dielectrophoretic trapping force versus electrothermal drag force on bio-particles.

3. Real-time electrochemical monitoring of ATP at graphene-modified electrodes”, B. Sanghavi, S. Sitaula, M. Griep, S. Karna, M. Ali, N. S. Swami* Anal. Chem. (2013), 85, 8158–8165 Journal Impact Factor =5.8:

We report on a competitive electrochemical detection system that is free of wash-steps and enables the real-time monitoring of adenosine triphosphate (ATP) over a five-log concentration range, with the ability to speed-up target binding kinetics by increasing capture probe concentration. This displacement based assay enables biomarker detection by using nanoparticle-immobilized receptors, thereby obviating the need for functionalization of microfluidic devices to enable biomarker recognition.

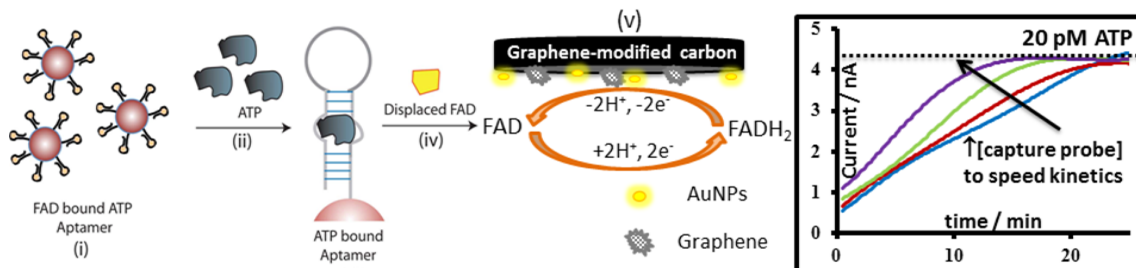


Fig. 3: Example sensing paradigm based on competitive displacement of pre-bound electroactive FAD From aptamer receptors for enabling monitoring of ATP through electrochemical detection.

4. “Electrokinetic preconcentration and detection of neuropeptides at patterned graphene-modified electrodes in a nanochannel”, Sanghavi, B. J., Varhue, W., Chávez, J. L., Chou, C. F., & Swami, N. S.*; Analytical chemistry, 86(9), 4120-4125. Impact Factor = 5.83;

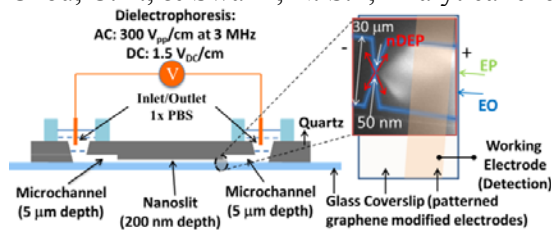


Fig. 4: Coupling electrokinetic enrichment of Neuropeptides with electrochemical detection.

Neuropeptides are vital to the transmission and modulation of neurological signals, with Neuropeptide Y (NPY) and Orexin A (OXA) offering diagnostic information on stress, depression, and neurotrauma. NPY is an especially significant biomarker, since it can be noninvasively collected from sweat, but its detection has been limited by poor sensitivity,

long assay times, and the inability to scale-down

sample volumes. Herein, we apply electrokinetic preconcentration of the neuropeptide onto patterned graphene-modified electrodes in a nanochannel by frequency-selective

dielectrophoresis for 10 s or by electrochemical adsorptive accumulation for 300 s, to enable the electrochemical detection of NPY and OXA at picomolar levels from subnanoliter samples, with sufficient signal sensitivity to avoid interferences from high levels of dopamine and ascorbic acid within biological matrices. Given the high sensitivity of the methodology within small volume samples, we envision its utility toward off-line detection from droplets collected by microdialysis for the eventual measurement of neuropeptides at high spatial and temporal resolutions.

5. “Quantifying spatio-temporal dynamics of biomarker pre-concentration and depletion in microfluidic systems by intensity threshold analysis”, A. Rohani, W. Varhue, Y.-H. Su, N. S. Swami; *Biomicrofluidics* (2014) 8, 052009. Journal Impact Factor =3.8

Microfluidic systems are commonly applied towards pre-concentration of biomarkers for enhancing detection sensitivity. Quantitative information on the spatial and temporal dynamics of pre-concentration, such as its position, extent and time evolution are essential towards sensor design for coupling pre-concentration to detection. Current quantification methodologies are based on the time evolution of fluorescence signals from biomarkers within a statically defined region of interest, which does not offer information on the spatial dynamics of pre-concentration and leads to significant errors when the pre-concentration zone is delocalized or exhibits wide variations in size, shape and position over time under the force field. We present a dynamic methodology for quantifying the region of interest by using a statistical description of particle distribution across the device geometry to determine the intensity thresholds for particle pre-concentration. This method is applied to study the delocalized pre-concentration dynamics under an electrokinetic force balance driven by negative dielectrophoresis, for aligning the pre-concentration and detection regions of neuropeptide Y, and for quantifying the polarizability dispersion of silica nano-colloids with frequency of the force field. We envision the application of this automated methodology on data from 2D images and 3D Z-stacks for quantifying pre-concentration dynamics over delocalized regions as a function of the force field.

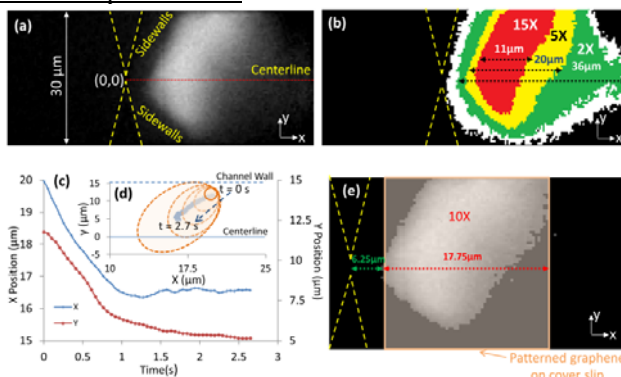


Fig. 5: Biomarker pre-concentration under force fields (a) over varying spatial (b) and temporal spreads (c & d) is quantified for alignment to sensing region (e).

6. “DNA Combing on Low-Pressure Oxygen Plasma Modified Polysilsesquioxane Substrates For Single-Molecule Studies”, K. K. Sriram, C.L. Chang, U. R. Kumar, C.F. Chou* (2014). *Biomicrofluidics* 2014, 8, 052102; [DOI: 10.1063/1.4892515](https://doi.org/10.1063/1.4892515). (IF: 3.771): Molecular combing and flow-induced stretching are the most commonly used methods to immobilize and stretch DNA molecules. While both approaches require functionalization steps for the substrate surface and the molecules, conventionally the former does not take advantage of, as the latter, the versatility of microfluidics regarding robustness, buffer exchange capability, and molecule manipulation using external forces for single molecule studies. Here, we demonstrate a simple one-step combing process involving only low-pressure oxygen (O₂) plasma modified polysilsesquioxane (PSQ) polymer layer to facilitate both room temperature microfluidic device bonding and immobilization of stretched single DNA molecules without molecular functionalization step. Atomic force microscopy and Kelvin probe force microscopy experiments

revealed a significant increase in surface roughness and surface potential on low-pressure O₂ plasma treated PSQ, in contrast to that with high-pressure O₂ plasma treatment, which are proposed to be responsible for enabling effective DNA immobilization. We further demonstrate the use of our platform to observe DNA-RNA polymerase complexes and cancer drug cisplatin induced DNA condensation using wide-field fluorescence imaging.

7. “Direct Optical Mapping of Transcription Factor Binding Sites on Field-stretched λ -DNA in Nanofluidic Devices”, K.K. Sriram, J.W. Yeh, Y.L. Lin, Y.R. Chang, C.F. Chou* (2014). *Nucleic Acids Research* 2014, 42, e85. DOI: 10.1093/nar/gku254 (IF: 8.808): Mapping transcription factor (TF) binding sites along a DNA backbone is crucial in understanding the regulatory circuits that control cellular processes. Here, we deployed a method adopting bioconjugation, nanofluidic confinement and fluorescence single molecule imaging for direct mapping of TF (RNA polymerase) binding sites on field-stretched single DNA molecules. Using this method, we have mapped out five of the TF binding sites of *E. coli* RNA polymerase to bacteriophage λ -DNA, where two promoter sites and three pseudo-promoter sites are identified with the corresponding binding frequency of 45% and 30%, respectively. Our method is quick, robust and capable of resolving protein-binding locations with high accuracy (~ 300 bp), making our system a complementary platform to the methods currently practiced. It is advantageous in parallel analysis and less prone to false positive results over other single molecule mapping techniques such as optical tweezers, atomic force microscopy and molecular combing, and could potentially be extended to general mapping of protein–DNA interaction sites.

8. “Low-Copy Number Protein Detection by Electrode Nanogap-Enabled Dielectrophoretic Trapping for Surface-enhanced Raman Spectroscopy and Electronic Measurements”, L. Lesser-Rojas, P. Ebbinghaus, G. Vasan, M.L. Chu, A. Erbe*, C.F. Chou* (2014). *Nano Letters* 2014, 14(5), 2242–2250. <http://dx.doi.org/10.1021/nl4046685>. (IF=13.025):

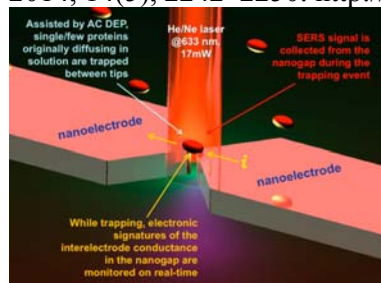


Fig. 6: Coupling DEP to detection

across the nanogaps, and fluorescence imaging show unambiguously the presence and characteristics of the trapped proteins. Our platform opens up a simple way for multifunctional low-concentration heterogeneous sample analysis without the need for target preconcentration.

9. “Tandem array of nanoelectronic readers embedded coplanar to a fluidic nanochannel for correlated single biopolymer analysis”, L. Lesser-Rojas, K. K. Sriram, K.T. Liao, S.C. Lai, P.C. Kuo, M.L. Chu, C.F. Chou* (2014). *Biomicrofluidics* 2014, 8, 016501. <http://dx.doi.org/10.1063/1.4861435> (IF=3.771): We have developed a two-step electron-beam lithography process to fabricate a tandem array of three pairs of tip-like gold nanoelectronic detectors with electrode gap size as small as 9 nm, embedded in a coplanar fashion to 60 nm deep, 100 nm wide, and up to 150 μ m long nanochannels coupled to a world-micro-nanofluidic interface for easy sample introduction. Experimental tests with a sealed device using

DNA-protein complexes demonstrate the coplanarity of the nanoelectrodes to the nanochannel surface. Further, this device could improve transverse current detection by correlated time-of-flight measurements of translocating samples, and serve as an autocalibrated velocimeter and nanoscale tandem Coulter counters for single molecule analysis of heterogeneous samples.

List of Publications and Significant Collaborations that resulted from your AOARD supported project: In standard format showing authors, title, journal, issue, pages, and date, for each category list the following:

a) papers published in peer-reviewed journals:

(i) K.T. Liao, M. Tsegaye, V. Chaurey, C.F. Chou, N. Swami. "Nano-constriction device for rapid protein pre-concentration in physiological media by electrokinetic force balance", *Electrophoresis* (2012), 33, 1958-1966. DOI: 10.1002/elps.201100707

(ii) V. Chaurey, A. Rohani, Y.-H. Su, W. Varhue, K.T. Liao, C. F. Chou, N. S. Swami. "Scaling down constriction-based dielectrophoresis for trapping nanoscale biomolecules in high conductivity media", *Electrophoresis* (2013), 34, 1097-1104. 10.1002/elps.201200456

(iii) B. J. Sanghavi, S. Sitaula, M. Griep, S. Karna, M. Ali, N. S. Swami. "Real-time electrochemical monitoring of ATP at graphene-modified electrodes", *Anal. Chem.* (2013), 85, 8158–8165. DOI: 10.1021/ac4011205

(iv) A. Rohani, W. Varhue, Y.-H. Su, N. S. Swami. "Quantifying spatio-temporal dynamics of biomarker pre-concentration and depletion in microfluidic systems by intensity threshold analysis", *Biomicrofluidics* (2014) 8, 052009. <http://dx.doi.org/10.1063/1.4897283>

(v) B. Sanghavi, W. Varhue, J. Chavez, C.F. Chou, N. S. Swami. "Electrokinetic preconcentration and detection of neuropeptides at patterned graphene-modified electrodes in a nano-channel device", *Anal. Chem.* (2014), 86, pp 4120–4125. DOI:10.1021/ac500155g

(vi) K. K. Sriram, C.L. Chang, U. R. Kumar, C.F. Chou* (2014). "DNA Combing on Low-Pressure Oxygen Plasma Modified Polysilsesquioxane Substrates For Single-Molecule Studies", *Biomicrofluidics* 2014, 8, 052102; DOI: 10.1063/1.4892515. (IF: 3.771)

(vii) K.K. Sriram, J.W. Yeh, Y.L. Lin, Y.R. Chang, C.F. Chou* (2014). "Direct Optical Mapping of Transcription Factor Binding Sites on Field-stretched λ -DNA in Nanofluidic Devices", *Nucleic Acids Research* 2014, 42, e85. DOI: 10.1093/nar/gku254 (IF: 8.808)

(viii) L. Lesser-Rojas, K. K. Sriram, K.T. Liao, S.C. Lai, P.C. Kuo, M.L. Chu, C.F. Chou* (2014). "Tandem array of nanoelectronic readers embedded coplanar to a fluidic nanochannel for correlated single biopolymer analysis", *Biomicrofluidics* 2014, 8, 016501. <http://dx.doi.org/10.1063/1.4861435> (IF=3.771)

(ix) L. Lesser-Rojas, P. Ebbinghaus, G. Vasan, M.L. Chu, A. Erbe*, C.F. Chou* (2014). "Low-Copy Number Protein Detection by Electrode Nanogap-Enabled Dielectrophoretic Trapping for Surface-enhanced Raman Spectroscopy and Electronic Measurements", *Nano Letters* 2014, 14(5), 2242–2250. <http://dx.doi.org/10.1021/nl4046685>. (IF=13.025)

b) papers published in non-peer-reviewed journals or in conference proceedings: None

c) conference presentations (Selected)

(i) B.J. Sanghavi, W. Varhue, A. Rohani, J. Chavez, C.-F. Chou, N.S. Swami; "Conformation-selective biomarker preconcentration by dielectrophoresis", MicroTAS 2014, San Antonio, USA

(ii) K.-T. Laio, N. S. Swami, C.-F. Chou. "Rapid monitoring of low abundance prostate specific antigen by protein nanoconstriction molecular dam." MicroTAS, Germany (2013). http://www.rsc.org/images/loc/2013/PDFs/Papers/471_0719.pdf

(iii) B.J. Sanghavi, W. Varhue, A. Rohani, J. Chavez, C.-F. Chou, N.S. Swami; "Electrokinetic

preconcentration and detection of neuropeptides”, AIP Advances in Micro/Nanofluidics, Academia Sinica, Taipei, Taiwan.

(iv) N. S. Swami, “Frequency selective trapping of biomarkers using conformation-specific aptamers”, Microscale Bioseparations: MSB 2013, Charlottesville, VA, March 2013.

(v) N.S. Swami, “Frequency-selective polarization of the electrical double-layer of nano-colloids”, American Electrophoresis Society Annual Meeting, AIChE, San Francisco, Nov 2013

(vi) N.S. Swami, “Coupling dielectrophoresis to ion concentration polarization for enhanced protein enrichment” Advances in Micro-Nanofluidics, AMN 2013, University of Notre Dame, May 2013

(vii) N.S. Swami, “Nano-slit device for dielectrophoretic enrichment of proteins”, ITP Separations, Baltimore, 2012.

d) manuscripts submitted but not yet published (None)

e) provide a list any interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work.

Collaborations with AFRL’s 711th Human Performance Wing: Nancy Kelley-Loughnane and Jorge Chavez (1 published paper and 2 manuscripts in progress)

DD882: No inventions disclosures (form submitted).

Important Note: Abstracts of refereed publications have been submitted above as part of “Results & Discussion”, and reprints are included as an appendix.

References

¹ USAF Chief Scientist in Technology Horizons, 15 May 2010.

² V. Polaskova, A. Kapur, A. Khan, M. P. Molloy and M. S. Baker. “High-abundance protein depletion: Comparison of methods for human plasma biomarker discovery”, *Electrophoresis* (2010), **31**, 471-482.

³ B. C. Giordano, D. S. Burgi, S. J. Hart and A. Terray. “On-line sample pre-concentration in microfluidic devices: A review”, *Analytica Chimica Acta* (2012) **718**, 11-24.

⁴ C. C. Lin, J. L. Hsu and G. B. Lee. “Sample preconcentration in microfluidic devices”, *Microfluid Nanofluid* (2011) **10** (3), 481-511.

⁵ K.T. Liao, M. Tsegaye, V. Chaurey, C.F. Chou, N. Swami. “Nano-constriction device for rapid protein pre-concentration in physiological media by electrokinetic force balance”, *Electrophoresis* (2012), **33**, 1958-1966.

⁶ V. Chaurey, A. Rohani, Y.-H. Su, W. Varhue, K.T. Liao⁺, C. F. Chou, N. S. Swami. “Scaling down constriction-based dielectrophoresis for trapping nanoscale biomolecules in high conductivity media”, *Electrophoresis* (2013), **34**, 1097-1104.

⁷ B. J. Sanghavi, S. Sitaula, M. Griep, S. Karna, M. Ali, N. S. Swami. “Real-time electrochemical monitoring of ATP at graphene-modified electrodes”, *Anal. Chem.* (2013), **85**, 8158–8165.

⁸ A. Rohani, W. Varhue, Y.-H. Su, N. S. Swami. “Quantifying spatio-temporal dynamics of biomarker pre-concentration and depletion in microfluidic systems by intensity threshold analysis”, *Biomicrofluidics* (2014) **8**, 052009.

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